

Effect of artemisinin combined with allicin on improving cardiac function, fibrosis and NF- κ B signaling pathway in rats with diabetic cardiomyopathy

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Myocardial fibrosis and inflammation cause cardiac hypertrophy, arrhythmias, and heart failure in diabetics, a leading cause of mortality. Since it's complicated, no drug treats diabetic cardiomyopathy. This research examined the effects of artemisinin and allicin on heart function, myocardial fibrosis, and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathway in diabetic cardiomyopathy rats. A total of 50 rats were separated into 5 groups, 10 of which were the control group. 40 rats received 65 μ g/g streptozotocin intraperitoneally. 37 of 40 animals fit the investigation. The artemisinin, allicin, and artemisinin/allicin groups each included nine animals. The artemisinin group received 75 mg/kg of artemisinin, the allicin group received 40 mg/kg of allicin, and the combination group received equal dosages of artemisinin and allicin gavage for four weeks. After the intervention, in each group cardiac functions, myocardial fibrosis, and NF- κ B signaling pathway protein expression were assessed. All of the examined groups had greater levels of LVEDD, LVESD, LVEF, FS, E/A, and the NF- κ B pathway proteins: NF- κ B p65 and p-NF- κ B p65 than the normal group, except for the combination group. Artemisinin and allicin did not vary statistically. Compared to the model group, the artemisinin, allicin, and combined groups showed various degrees of improvement from the pathological pattern, with more intact muscle fibers, neater arrangement, more normal cell morphology, artemisinin and allicin alleviated cardiac dysfunction and decreased myocardium fibrosis in diabetic cardiomyopathy rats by inactivating the NF- κ B signaling cascade.

Keywords: artemisinin, allicin, diabetic cardiomyopathy, cardiac function, myocardial fibrosis, nuclear factor- κ B

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Abbreviations: DC, Diabetic cardiomyopathy; DM, Diabetes mellitus; FAC, Fractional area change; FS, Fractional shortening; H&E, Hematoxylin and Eosin; iNOS, Inducible nitric oxide synthase; I κ B- α , Inhibitory protein kappa B alpha; LVEDD, Left ventricular end-diastolic internal diameter; LVEF, Left ventricular ejection fraction; LVESD, Left ventricular end-systolic internal diameter; MOG, Myelin oligodendrocyte glycoprotein; NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells

INTRODUCTION

Diabetic cardiomyopathy (DC) is a primary cause of mortality in people with diabetes mellitus (DM) and is characterized by myocardial fibrosis and myocardial inflammation, with cardiac hypertrophy, arrhythmias, and

heart failure as the main manifestations (Dillmann, 2019). DC is complex and involves several factors, so there is no specific drug to treat this disease. Allicin, the active component of garlic (*Allium sativum*), has been clinically shown to have an anti-cardiac fibrosis effect, and it has been reported that allicin is effective in diabetic cardiomyopathy, but a single drug may not completely inhibit the progression of diabetic cardiomyopathy (Liu *et al.*, 2021). Artemisinin belongs to the extract of the Chinese herb *Artemisia annua*, which was found to attenuate myocardial fibrosis and ventricular remodeling *via* inhibition of the cascade of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Gu *et al.*, 2012).

Artemisinin has recently been found to decrease inducible nitric oxide synthase (iNOS) generation and NF- κ B stimulation in human astrocytoma T67 cells. SM933, an artemisinin derivative, has been reported to suppress NF- κ B activity by blocking its breakdown through overexpression of its inhibitory protein kappa B alpha (I κ B- α) and MOG-reactive splenocytes (Zhang *et al.*, 2020). Combined, these findings suggest that artemisinin may have a role in immunological modulation and inflammation reduction. The effects of the anti-inflammatory properties of artemisinin on microglial activation are not clear (Kim *et al.*, 2019). The current study was carried out to assess the maximum extent of inhibition of diabetic cardiomyopathy development and the reduction of fibrosis and regulation of the NF- κ B cascade by a combination of allicin and artemisinin.

MATERIALS AND METHODS

Study area

The present study was carried out at the Chengde Medical College, College of Traditional Chinese Medicine from January to April 2022.

Main reagents

Streptozotocin (Beijing Ita Biotechnology Co., Ltd.), artemisinin (Nanjing Guangrun Biological Products Co., Ltd.), allicin (Zhengda Tianqing Group Co., Ltd.), NF- κ B p65, p-NF- κ B p65 antibody (primary) (Abcam, USA).

Animals study

In this study, 50 SPF pure standard Wistar male rats (6–8 weeks old, 220 \pm 20 g) were received from Hangzhou Qizhen Research Animal Technology Co., China. In this study, 7 days of acclimatization under constant temperature (22 \pm 2 $^{\circ}$ C), humidity (55 \pm 5%), and artificial

12 hrs light and dark cycle. All were free to move, drink, and take food. Chengde Medical College, College of Traditional Chinese Medicine ethics committee gave its approval to carry out this investigation (approval number: FEH83456).

Modeling and intervention

In total, 50 animals (n=50, 16-week-old) were used in this study, of which 10 were chosen as the normal group. The remaining 40 animals were given a single injection of 65 g/kg streptozotocin intraperitoneally, and their blood glucose levels were evaluated one week later. If the blood glucose value was ≥ 16.7 mmol/L and there were polyuria, polydipsia, and polyphagia, then the modelling of diabetes was successful (Togashi *et al.*, 2016). Streptozotocin was administered to 40 animals, and these animals were successfully established as a model of diabetes. They were fed by standard fodder until 16 weeks, and 3 animals were detected by ultrasound as not having developed cardiomyopathy, and the remaining animals (n=37) were successfully established as diabetic cardiomyopathy models.

Experimental design

- Normal group: without treatment (n=10);
- Model group: saline treatment (10 μ L) (n=10);
- Artemisinin group: 75 mg/kg of artemisinin (n=9);
- Alicin group: 40 mg/kg of alicin (n=9);
- Artemisinin and alicin groups: 75 mg/kg of artemisinin+ 40 mg/kg of alicin (n=9).

All of these groups were treated continuously for 4 weeks, and the changes in experimental indexes in each group were observed after 20 weeks.

Measurement of cardiac function

After the last pharmacological intervention, echocardiographic parameters were taken by using a 7.5-MHz transducer (Toshiba Co., Japan) in each group to determine changes in cardiac function, and left ventricular end-diastolic internal diameter (LVEDD), left ventricular end-systolic internal diameter (LVESD), left ventricular ejection fraction (LVEF), and short-axis shortening (FS) measurements were utilized to examine alteration in cardiac function, maximum mitral valve velocity in early dilatation (E), and maximum atrial systolic velocity (A) ratio. Echocardiographic measures were collected from grayscale M-mode images captured in the parasternal short-axis perspective at the mid-papillary level, as well as from B-mode images recorded in the parasternal long- and short-axis views. End-diastolic diameter (LVEDD), end-systolic diameter, anterior and posterior wall thicknesses, FS, wall thickening, fractional area change (FAC), end-systolic and end-diastolic volumes, EF, and LV mass were all traditional LV measures.

Table 1. Comparison of cardiac function in each group ($\bar{x} \pm s$)

Group	LVEDD (mm)	LVESD (mm)	LVEF (%)	FS (%)	E/A
Normal group (n=10)	5.70 \pm 0.11	2.20 \pm 0.08	78.75 \pm 2.31	57.54 \pm 2.10	2.23 \pm 0.12
Model group (n=10)	7.00 \pm 0.23*	4.65 \pm 0.10*	63.32 \pm 2.45*	36.55 \pm 1.04*	1.30 \pm 0.09*
Artemisinin group (n=9)	6.51 \pm 0.11**	4.00 \pm 0.14**	66.75 \pm 2.11**	40.62 \pm 1.33**	1.55 \pm 0.10**
Alicin group (n=9)	6.48 \pm 0.11**	4.03 \pm 0.16**	66.98 \pm 2.09**	40.55 \pm 1.54**	1.58 \pm 0.11**
Combined group (n=9)	6.00 \pm 0.10**& Δ	2.98 \pm 0.09**& Δ	73.42 \pm 2.76**& Δ	53.39 \pm 2.13**& Δ	2.07 \pm 0.13**& Δ

Note: "**" indicates significant comparison with the normal group; "*" indicates significant comparison with the model group; "&" indicates significant comparison with the artemisinin group; " Δ " indicates meaningful comparison with the alicin group.

Observation of myocardial fibrosis

After the completion of cardiac function measurement, rapid sacrifice of rats, preparation of myocardial tissue, tissue sectioning and then Hematoxylin and Eosin (H&E) staining, Masson staining, and microscopic observation of myocardial fibrotic lesions were performed in each group. After soaking in 4% neutral formalin solution for 24 h, all tissues were promptly cut and stained with Masson's trichrome kit (Sigma-Aldrich, USA). On each slide, three sections were examined, and each region was divided into 100 squares. Collagen points (blue stains) were scored as 1 (existent) or 0 (absence). The findings are presented as a proportion of the total region covered by fibrosis.

NF- κ B signaling cascade protein expression measurement

The expression of the NF- κ B signaling pathway proteins NF- κ B p65 and p-NF- κ B p65 was determined using the Western blot technique. Myocardial tissue was obtained, 80 μ L of lysis buffer was added, tissue protein was extracted, and 50 mg of the resulting protein were mixed with 2 \times SDS loading buffer. Following electrophoresis, 15% denaturing gels were prepared according to the method of Summer and others (Summer *et al.*, 2009). In this study, membrane transfer, and antisera binding, NF- κ B p65 and p-NF- κ B p65 primary antibodies diluted at a 1:1000 ratio in TBST buffer were used, and the cells were maintained at 4°C. After 1 day, the TBST buffer was washed, an HRP-labeled secondary antibody was applied, and the membrane was rinsed. The diaminobenzidine technique was utilised for colour development, and GAPDH was employed as an internal reference to get the protein expression of NF- κ B p65 and p-NF- κ B p65.

Statistical analysis

The SPSS 22.0 statistical tool (V13.0; SPSS, Inc., USA) was employed to evaluate the data, and one-way ANOVA was utilised to examine variance between groups. For two-by-two comparisons among groups, the LSD-t test was utilized. After natural logarithm transformation and non-parametric test, the data did not adhere to normally distributed and were expressed as M (Qn) for data comparison. The threshold of significance was fixed at $p < 0.05$.

RESULTS

Assessment of cardiac function in each group

The model group, artemisinin group, alicin group, and combined group had greater LVEDD, LVESD,

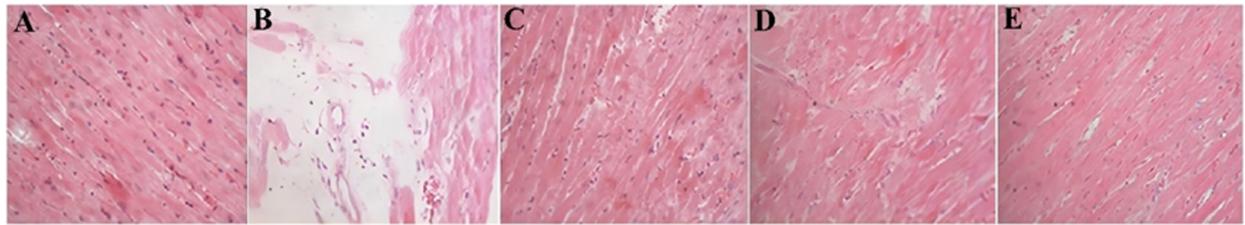


Figure 1. HE staining observation of myocardial tissue ($\times 200$)

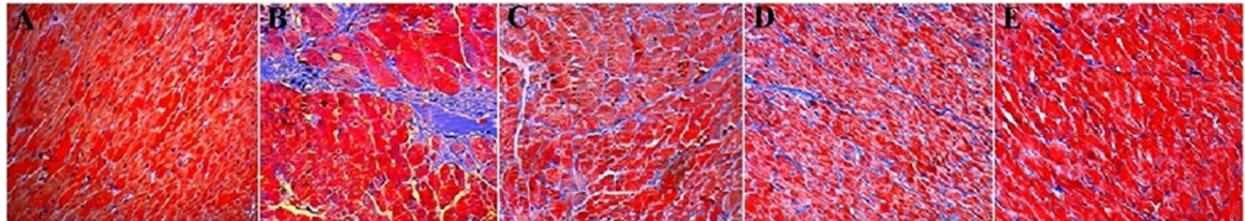


Figure 2. Myocardial tissue Masson staining observation map ($\times 200$)

Note: A, Normal group; B, Model group; C, Artemisinin group; D, Allicin group; E, Combination group

LVEF, FS, E/A, etc. and cardiac function indexes than the normal control, and the variation was statistically relevant ($p < 0.05$). The cardiac function indicators LVEDD, LVESD, LVEF, FS, and E/A were decreased in the artemisinin group, allicin group, and combined group compared to the model group, and the change was statistically significant ($p < 0.05$). The cardiac performance parameters LVEDD, LVESD, LVEF, FS, and E/A were lower in the combination group compared to artemisinin and allicin groups, with significant variations ($p < 0.05$). There was no discernible difference between the groups treated with artemisinin and allicin in terms of LVEDD, LVESD, LVEF, FS, E/A, and the cardiac function indexes ($p > 0.05$). For further details, see Table 1.

Myocardial fibrosis observation

Microscopic analysis of animal cardiac tissues revealed that myocardium cells in the normal group were in a healthy condition, but a minor quantity of collagen fibres and no pathological alterations were observed. The collagen fibres multiplied and structured themselves into a network in the model group, which led to many fibrous scar formations, cardiomyocyte enlargement, uneven staining, nucleus consolidation, fragmentation, and even disappearance. Myocardial fibrosis was dramatically reduced in each treatment group as compared to the model group, cell morphology was gradually recovered and staining was more uniform, and myofibers tended to be intact, in comparison to the artemisinin and allicin groups, pathological alterations were more clearly restored in the combination group (Figs 1 and 2).

Comparison of NF- κ B signaling pathway protein expression among groups

The model group, artemisinin group, allicin group, and combined group had increased expression of NF- κ B p65 and p-NF- κ B p65, the NF- κ B signaling pathway proteins, than the normal group, in which the variation was statistically relevant ($p < 0.05$). The proteins of the NF- κ B signaling pathway, NF- κ B p65 and p-NF- κ B p65, were expressed at lower levels in the artemisinin, allicin, and combination groups than in the model group, and the difference was statistically significant ($p < 0.05$). The combined group had lower levels of

NF- κ B p65 and p-NF- κ B p65, the NF- κ B signaling pathway proteins, than the artemisinin and allicin groups, and

Table 2. Protein expression of the NF- κ B signaling pathway in different groups

Group	NF- κ B p65	p-NF- κ B p65
Normal group (n=10)	0.35 \pm 0.04	0.56 \pm 0.07
Model group (n=10)	1.89 \pm 0.22*	1.93 \pm 0.24*
Artemisinin group (n=9)	1.03 \pm 0.15 [#]	1.12 \pm 0.15 [#]
Allicin group (n=9)	1.06 \pm 0.12 [#]	1.14 \pm 0.13 [#]
Combination group (n=9)	0.70 \pm 0.08 ^{*#Δ}	0.88 \pm 0.09 ^{*#Δ}

Note: "*" indicates significant comparison with the normal group; "*" indicates significant comparison with the model group; "[#]" indicates significant comparison with the artemisinin group; " ^{Δ} " indicates significant comparison with the allicin group.

the variation was statistically significant ($p < 0.05$). There were no substantial changes in the expression of NF- κ B, p65 and p-NF- κ B, p65, the NF- κ B signaling pathway proteins, between the artemisinin and allicin groups ($p > 0.05$). More data was shown in Table 2.

DISCUSSION

Allicin causes cell death and limits cell growth in mammalian cell lines, including cancer cells, by reacting redox with thiol groups in glutathione and proteins, inhibiting bacterial and fungal proliferation or killing them completely in a dose-dependent manner. Allicin has a number of health-promoting qualities, including effects on cholesterol and blood pressure that are beneficial to the cardiovascular system. According to Kuo and others (Kuo *et al.*, 2013), allicin seems to prevent high-glucose-induced cardiomyocyte apoptosis *via* reducing NADPH oxidase-related ROS and its subsequent NK/NF- κ B signaling, and may have therapeutic potential for diabetic cardiomyopathy.

Artemisinin belongs to the sesquiterpene lactones with peroxy bridges, which were first used in the treatment of malaria and have anticancer, antiviral, antiparasitic *in vitro* and *in vivo* anti-inflammatory properties, in addition

to their antimalarial effects (Ma *et al.*, 2020; Talman *et al.*, 2019). With extensive clinical studies on artemisinin, it has been found that it can alter fibrosis *via* several pathways, such as TGF, MAPK, Wnt/catenin, PI3K/AKT/mTOR, FRX and Notch, NF- κ B signaling pathways, as well as BMP-7 and cellular autophagy, which contribute to the anti-fibrotic process, as does its anti-inflammatory effect (Li *et al.*, 2019; Dolivo *et al.*, 2021).

The results of this paper showed that compared with artemisinin and allicin alone, the cardiac function of rats with the combined administration of both interventions improved more significantly. In HE and Masson staining, it was found that the myocardium of rats with diabetic cardiomyopathy showed obvious fibrosis, as well as the severity of cardiac fibrosis was relieved after the intervention of artemisinin and allicin, the myocardial fibers were more intact and neatly arranged, the cell morphology tended to be normal, and the staining was more homogeneous, but the improvement was more obvious with artemisinin combined with allicin, indicating that the myocardial damage was more serious in the occurrence of diabetic cardiomyopathy, and the cardiac function of rats with artemisinin and allicin administration improved significantly and the myocardial fibrosis was relieved.

The myocardial structure is altered when the animal cells are under prolonged hyperglycemia, and the NF- κ B signaling cascade is initiated during this process. NF- κ B is secreted by vascular endothelium, vascular smooth muscle cells, etc.; this is a nuclear transcription factor that degrades IB with the help of inflammatory mediators, turns on and activates NF- κ B, and when NF- κ B is activated it can increase the binding to nuclear DNA to some extent, which in turn acts on cardiomyocyte behavior and leads to apoptosis (Youssef *et al.*, 2021; Yao *et al.*, 2021; Tang *et al.*, 2018). In addition, NF- κ B activates inflammatory cytokines including interleukins and tumor necrosis factor and further circulates to activate NF- κ B itself, amplifying the local cascade response in this circulatory pattern and promoting the progression of myocardial injury (Yang *et al.*, 2018; Jin *et al.*, 2020; Ni *et al.*, 2020). The effects on the NF- κ B signaling pathway in diabetic cardiomyopathy rats were examined in this work. Transartemisinin combined with allicin reduced NF- κ B p65 expression and NF- κ B p65 phosphate activation level in diabetic cardiomyopathy rats, which inhibited over-activation of the NF- κ B signaling pathway, enhanced cardiac function, and reduced the degree of myocardial fibrosis, according to the findings. In summary, the present study found a rat model of diabetic cardiomyopathy and confirmed that artemisinin combined with allicin intervention improved cardiac function, myocardial fibrosis and NF- κ B signaling pathway in diabetic cardiomyopathy rats, implying that artemisinin conjunction with allicin intervention may perform a role in improving cardiac functions and inhibiting myocardial fibrosis *via* the NF- κ B signaling pathway, which has positive implications. However, this study also has its limitation in that the development of diabetic cardiomyopathy is associated with multiple factors and involves several mechanisms and further studies are needed regarding whether both act *via* other pathways.

CONCLUSION

Myocardial fibrosis and inflammation cause cardiac hypertrophy, arrhythmias, and heart failure in diabetics, a leading cause of mortality. Since it is complicated, no

drug treats diabetic cardiomyopathy. Therefore, there is a need to find a solution to this problem. This study was carried out to determine the effectiveness of artemisinin and allicin on heart function, myocardial fibrosis, and the nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway in diabetic cardiomyopathy rats. According to the findings of the study, artemisinin combined with allicin improved cardiac dysfunction and reduced myocardial fibrosis in rats with diabetic cardiomyopathy, and both may act *via* promoting the inactivation of NF- κ B signaling cascade.

Declarations

Acknowledgements. The authors acknowledge the facilities offered by the department higher authorities.

Data availability. The information used to justify this investigation is provided upon request.

Conflicts of interest. The authors state that they do not have any conflicts of interest.

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